Appendix B2

SHORT COMMUNICATION

Discovery of Three Novel G-Protein-Coupled Receptor Genes

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We report here the molecular cloning, tissue distribution, and chromosomal localization of novel genes encoding G-protein-coupled receptors (GPCRs). A search of a mouse database of expressed sequence tags revealed an EST partially encoding a GPCR, which was used to screen a mouse genomic library to obtain the translational open reading frame (ORF). The resultant clone, GPR27, contained an intronless ORF, encoding a receptor of 379 amino acids. In an alternate strategy, human genomic DNA was subjected to polymerase chain reaction (PCR) amplification, using degenerate oligonucleotides based on GPR1. Two PCR products partially encoding GPCRs were isolated and used to screen a genomic library to obtain the translational ORF. One of the resultant clones, GPR30, contained an intronless ORF encoding a receptor of 375 amino acids. The other clone, GPR35, also contained an intronless ORF encoding a receptor of 309 amino acids. Transcripts corresponding to GPR27 and GPR30 were detected in several areas of human and rat CNS, while GPR35 expression was detected only in the rat intestine. Through fluorescence *in situ* hybridization analysis the gene encoding GPR30 was localized to chromosome 7p22 and GPR35 to chromosome 2q37.3.

G-protein-coupled receptors (GPCRs) are membrane proteins and are the targets for a wide array of endogenous molecules encompassing small molecule neurotransmitters, glycoprotein hormones, chemokines, odorants, and neuropeptides, and they are also affected by light. The receptors and their ligands mediate a range of physiological processes, including feeding, reward, learning, mood, pain, movement, vision, smell, and chemotaxis. Many of the genes encoding GPCRs that bind known ligands have been cloned; however, molecular cloning techniques have identified a growing list of genes encoding GPCRs for which the endogenous ligands are not known; these are referred to as orphan receptors. At least 50 novel genes encoding orphan GPCRs have been identified and have been published or submitted to GenBank. We have reported the cloning of 20 novel genes encoding orphan receptors, many of which are expressed in the central nervous system, including APJ (13); GPR1, GPR2, and GPR3 (11); GPR4, GPR5, and GPR6 (6); GPR7 and GPR8 (14); GPR9, GPR10, and GPR14 (12); GPR15 (7); GPR19 (15); GPR20, GPR21, GPR22, and GPR23 (16); GPR24 (9); and GPR25 (8). Both GPR1 and GPR15 have recently been identified as coreceptors for simian immunodeficiency virus infection (2, 3). In our continuing effort to identify novel genes encoding GPCRs, we now report the cloning and the tissue distribution of GPR27, GPR30, and GPR35 and the chromosomal localization of GPR27 and GPR35. GPR30 and GPR35 were obtained using oligonucleotides based on the sequence of GPR1.

We queried the dbEST maintained by the NCBI with the amino acid sequence of the dopamine D4 receptor for cDNA sequences encoding novel GPCRs, as previously described (15). Several sequences encoding novel GPCRs were identified, one of which was requested from the IMAGE Consortium (10) and is reported here. This EST (clone ID 402354; GenBank Accession No. W78348), was predicted to contain a partially encoded novel mouse GPCR from TM 5 to the stop codon and also the 3′-untranslated region. This cDNA was used to screen a mouse genomic library, under conditions described previously (11), ultimately leading to the isolation of a 10-kb *Eco*RI and an 8-kb *Hin*dIII fragment. It was determined that these fragments contained the gene GPR27, with a consensus sequence for an initiation methionine preceded by an upstream in-frame stop codon and followed by an open reading frame (ORF) encoding a protein of 379 amino acids (Fig. 1). The amino acid sequence of the receptor

Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AF027955–AF027957.

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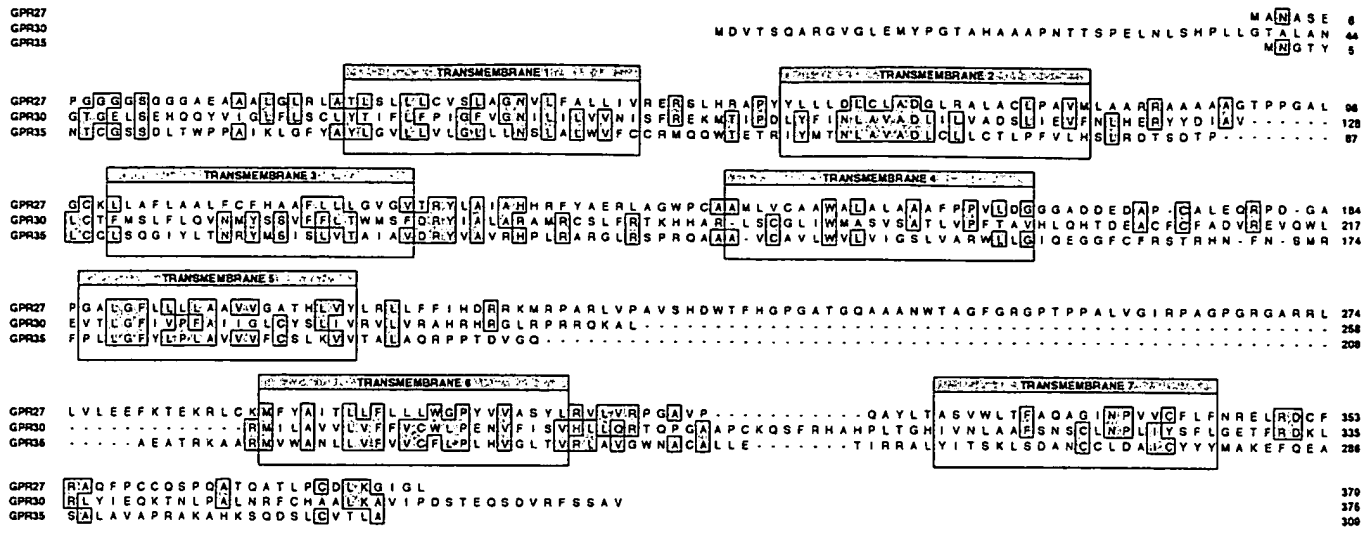

FIG. 1. Alignment of the deduced amino acid sequences of the receptors encoded by GPR27, GPR30, and GPR35. Boxed and shaded areas indicate identities between any two of the three sequences. The putative transmembrane domain regions are indicated. Gaps (−) have been introduced to maximize the alignment between the sequences. The single-letter amino acid code is used to represent amino acids. Sequence data have been deposited with the GenBank database under Accession Nos. AF027955 (GPR27), AF027956 (GPR30), and AF027957 (GPR35).

encoded by GPR27 was compared with sequences within the GenBank database by performance of a BLAST search (1). The amino acid sequence encoded by GPR27 showed highest identity with the dopamine D4 receptor (≈28% overall) and between 21 and 25% overall identity with other members of the dopamine and serotonin receptor families, including dopamine D5, D2, and D3 and serotonin 5-HT_{2A}, 5-HT_{2C}, 5-HT_7, and 5-HT_6 receptors. The receptor encoded by GPR27 lacks several amino acid residues considered critical for dopamine or serotonin binding, namely, an aspartic acid in TM3 and serine residues in TM5.

We reported the cloning of the gene GPR1 encoding a receptor (11), showing similarity to the C5a anaphylatoxin receptor (5). GPR1 was mapped by FISH analysis to chromosome 15, region q21.6, and also cross-hybridized with a locus on chromosome 2 (11). In an attempt to identify the nature of this cross-hybridization and also to isolate novel genes related to GPR1, human genomic DNA was subjected to PCR amplification using degenerate oligonucleotides designed based on sequences encoding TM2 [5′-CTCAA(T/C)(C/T)T(A/G)-GC(G/C)AT(A/T)GC (G/C)GA] and TM7 [5′-TAAAG-(C/G)ATGGGGTTCA(A/T)GCA(G/A)C(A/T)(A/G)TT] of the receptor encoded by GPR1 and the C5a anaphylatoxin receptor, under the same conditions as previously described (12). This resulted in the identification of three PCR products, each encoding a unique GPCR. We have previously reported one of these products, corresponding to the gene encoding the orphan receptor GPR14 (12), while the other two are described here. One of these fragments was used to screen a human genomic library leading to the isolation of a 2.5-kb *Sac*I and a 3-kb *Xho*I fragment. The gene GPR30 contained a consensus sequence for an initiation methionine followed by an ORF encoding a protein of 375 amino acids (Fig. 1). A comparison of the amino acid sequence encoded by GPR30 with sequences in the GenBank database revealed that it had been identified by two other groups (4, 17), while this work was in progress. The amino acid sequence encoded by GPR30 showed highest identity with members of the chemoattractant receptor family, namely, formylpeptide receptor FPRL1 (≈32% overall identity) and formylpeptide-like receptor FPRL2 (≈32% overall identity), and with chemokine receptor CXCR1 (≈29% overall identity), suggesting that the endogenous ligand may be a chemokine.

The second PCR fragment was used to screen a human genomic library leading to the isolation of a 2-kb *Kpn*I–*Cla*I fragment. The gene GPR35 revealed a consensus sequence for an initiation methionine preceded by an in-frame upstream stop codon followed by an ORF encoding a protein of 309 amino acids (Fig. 1). The amino acid sequence encoded by GPR35 showed highest identity with the deduced amino acid sequence of the orphan receptors encoded by GPR23 (≈32% overall identity) and HM74 (≈30% overall identity), followed by several members of the P2Y receptor family, including $P2Y_1$, and $P2Y_4$, (≈29% overall identity). Although both GPR30 and GPR35 were isolated with oligonucleotides derived from GPR1, the receptors encoded by each of these genes shared very little sequence identity (less than 26%). Also, the receptor encoded by GPR27 shares very little sequence identity with the receptors encoded by GPR30 and GPR35 (less than 24%).

Northern blot analysis of poly(A) RNA isolated from various tissues revealed expression of GPR27 mRNA in rat whole brain, hippocampus, striatum, frontal cortex, thalamus, pons, and hypothalamus (Fig. 2A). A lower molecular weight transcript was detected in all regions

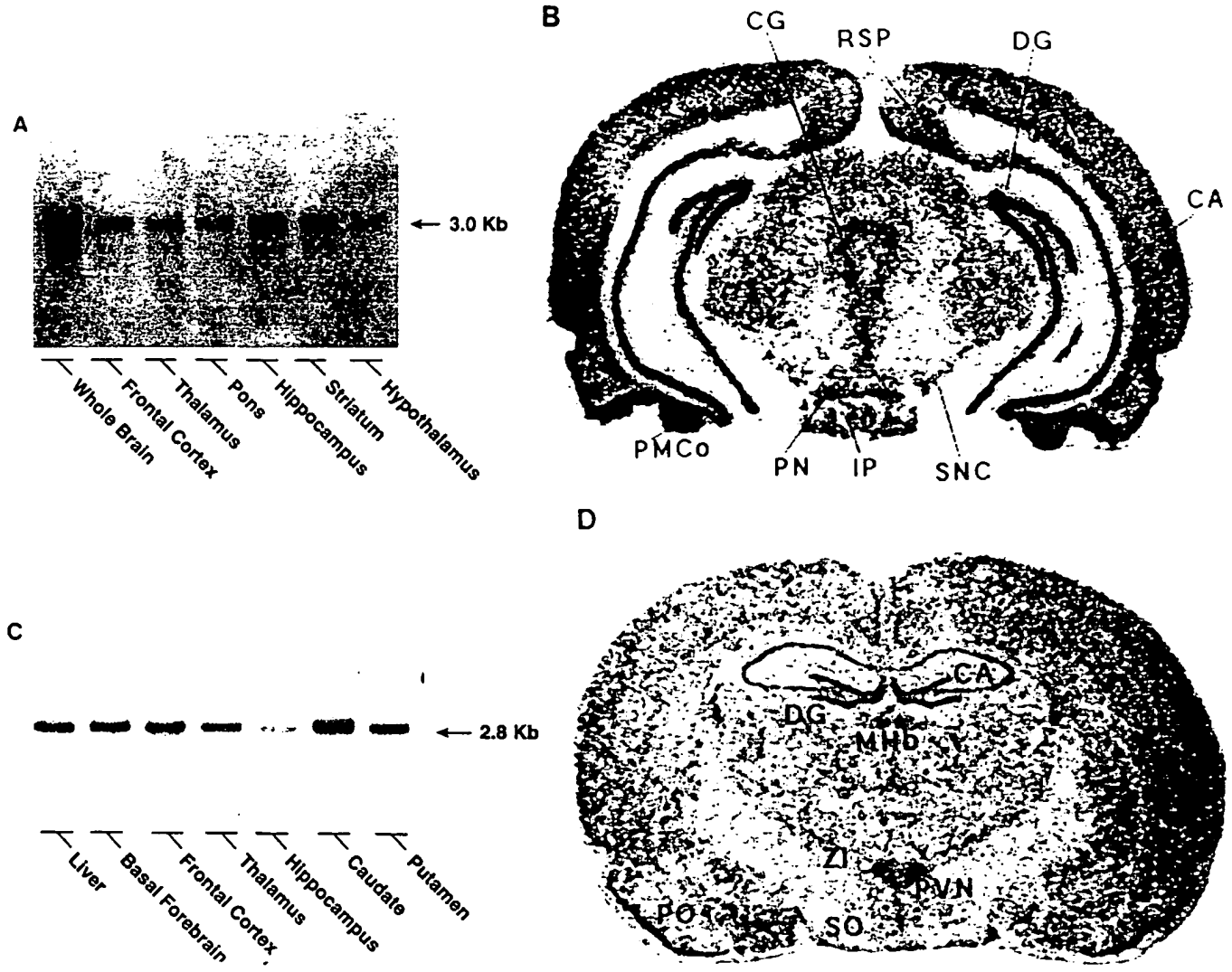

FIG. 2. Tissue distribution analyses of GPR27 and GPR30. Northern blots of (**A**) GPR27 in rat and (**C**) GPR30 in human showing mRNA transcripts from various tissues. 5 μg of poly(A)$^+$ RNA was loaded per lane and probed with radiolabeled fragments encoding each gene, under conditions previously described (11). The molecular size is indicated on the right. Autoradiograms of coronal sections of rat brain showing the localization of the mRNA for these genes are shown in representative sections at levels relative to bregma at (**B**) −5.8 mm for GPR27 and (**D**) −1.8 mm for GPR30, according to the coordinates by Paxinos and Watson (18). *In situ* hybridization experiments were performed as previously described (19). (CA, hippocampal area of Ammon's horn; CG, central gray; DG, dentate gyrus; IP, interpeduncular nucleus; MHb, medial habenular nucleus; PMCo, posteromedial cortical amygdaloid nucleus; PN, paranigral nucleus; PO, primary olfactory cortex; PVN, paraventricular nucleus of hypothalamus; RSP, retrosplenial area of cortex; SNC, substantia nigra, compacta; SO, supraoptic hypothalamic nucleus; ZI, zona incerta.

examined, except the hypothalamus (Fig. 2A). GPR30 mRNA expression was detected in the human liver, basal forebrain, frontal cortex, thalamus, hippocampus, caudate, and putamen (Fig. 2C). GPR35 expression was detected in the rat intestine, but not in the heart, spleen, liver, lung, ovary, kidney, or whole brain (data not shown) and was not detected in human caudate-putamen, thalamus, frontal cortex, pons, cortex, midbrain, medium pons, lung, or adrenal.

In situ hybridization experiments on rat brain sections were performed for GPR27 to obtain a more comprehensive brain expression pattern. GPR27 mRNA was present in cerebral cortex with the highest levels in piriform cortex. Moderate amounts were present in anterior cingulate, frontoparietal, and somatosensory areas of cortex, but with the signal concentrated in layers 1 and 6. GPR27 mRNA was present diffusely throughout the caudate putamen and nucleus accumbens, with very dense expression in the olfactory tubercle, the nucleus of the diagonal band, and the islands of Calleja. Several hypothalamic nuclei contained GPR27 mRNA, with high levels in suprachiasmatic, supraoptic, ventromedial, paraventricular, and arcuate nuclei. The medial habenular nucleus and the paraventricular nucleus of thalamus expressed moderately high amounts of GPR27 mRNA, and somewhat lesser amounts were visualized in other thalamic, certain amygdaloid, and septal nuclei. Certain amygdaloid nuclei and the hippocampal formation showed dense expression in the CA1, CA2, and CA3 fields of Ammon's

horn and in the dentate gyrus, with moderate expression in the central gray and interpeduncular nucleus (Fig. 2B).

GPR30 was also expressed in very discrete areas of the brain. The highest expression was in primary olfactory cortex, olfactory tubercle, nucleus of the lateral olfactory tract, hippocampal areas of Ammon's horn, and dentate gyrus (Fig. 2D). GPR30 mRNA was also densely concentrated in many hypothalamic nuclei, such as the paraventricular, supraoptic, arcuate, and suprachiasmatic nuclei. The signal detected in caudate nucleus and cortex was diffuse and much less abundant.

To assign the genes encoding GPR30 and GPR35 to a human chromosome, fluoresence *in situ* hybridization (FISH) analysis was performed on metaphase chromosomes prepared from lymphocytes using a biotinylated phage probe (≈20 kb) encoding either GPR30 or GPR35, as previously described (11). For each probe a total of 100 mitotic figures were analyzed; in the case of GPR30, 94 specifically bound the probe, and for GPR35, 96 specifically bound the probe. DAPI banding was performed to assign the signals to a particular chromosome, and the signals were further localized by superimposing the photographs from the FISH mapping and the DAPI staining (data not shown). As a result we were able to assign the gene encoding GPR30 to chromosome 7, region p22, and that encoding GPR35 to chromosome 2, region q37.3.

Future experiments will attempt to determine the endogenous ligands that bind to the receptors encoded by each respective gene, which will help elucidate a physiological role for these receptors.

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